

Assessment of antioxidant properties of alpha-keto acids in vitro and in vivo

Maria M. Bayliak¹  · Maria P. Lylyk¹ · Oksana M. Vytvytska¹ · Volodymyr I. Lushchak¹

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Abstract The antioxidant properties of three alpha-keto acids, pyruvate, oxaloacetate and alpha-ketoglutarate were tested in vitro and in vivo. In vitro, the keto acids demonstrated a good H₂O₂-scavenging activity, but were less effective as scavengers of free radicals or reductants of oxidized compounds. Alpha-ketoglutarate was a more effective H₂O₂ scavenger as well as HO[•] in Fe³⁺-ascorbate-EDTA-H₂O₂ system than other keto acids. All keto acids did not demonstrate iron-chelating activity. It was supposed that ability of keto acids to inhibit HO[•] production in the Fenton reaction could be mainly conditioned by their H₂O₂-scavenging activity. In vivo experiments, the addition of alpha-keto acids to the incubation medium significantly increased the resistance of exponentially growing yeast *Saccharomyces cerevisiae* cells to H₂O₂, transition metal ions (Fe²⁺), but not to menadione, a superoxide anion-generating compound. Similar results were obtained on fruit fly *Drosophila melanogaster*. In particular, co-treatment with alpha-ketoglutarate enhanced resistance of adult flies to H₂O₂ and did not prevent menadione-induced death of flies. Collectively, the results obtained indicate that exogenous alpha-keto acids as antioxidants are able efficiently to protect living organisms against stressors in which effects are mediated by H₂O₂. Therefore, they can be considered as available natural supplements to alleviate the harmful effects of reactive oxygen species.

Keywords Alpha-ketoglutarate · Oxaloacetate · Pyruvate · H₂O₂ scavenging · *Saccharomyces cerevisiae* · *Drosophila melanogaster*

Abbreviations

ABTS	2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
AKG	Alpha-ketoglutarate
KA/KAs	Keto acid/keto acids
OA	Oxaloacetate
ROS	Reactive oxygen species
TAA	Total antioxidant activity
TCA	Trichloroacetic acid

Introduction

Reactive oxygen species (ROS), such as superoxide anion radical (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radical (HO[•]), are normal by-products of aerobic cellular metabolism, and their production is enhanced by environmental stressful factors. Playing important physiological functions in cellular signaling at low levels, ROS become dangerous for cells at high levels and may cause wide-ranging damage to biomolecules that can eventually lead to cell death. To protect themselves against these damages, cells have effective defense mechanisms, including antioxidant enzymes and non-enzymatic free radical scavengers, to maintain ROS at certain steady-state level [1]. When ROS production exceeds the antioxidant capacity, cells undergo an oxidative stress characterized by increased oxidative processes and accumulation of oxidatively damaged proteins, lipids and DNA. Oxidative stress is a common hallmark accompanying various stress challenges, as well as aging and numerous diseases, including cancer, cardiovascular and neurodegenerative disorders [2–4].

✉ Maria M. Bayliak
bayliak@ukr.net

¹ Department of Biochemistry and Biotechnology, Vasyl Stefanyk Precarpathian National University, 57 Shevchenko Str., Ivano-Frankivsk 76018, Ukraine

The utilization of different antioxidant molecules with emphasis on natural antioxidants is one of the effective approaches to minimize deleterious effects of oxidative stress. The protective effects against oxidants are well documented for vitamins C, E and β -carotene, as well as many plant phenolic compounds [5]. It has been also reported that some organic keto acids which are normal intermediates of cellular metabolism may possess antioxidant properties. In particular, food supplementation with alpha-ketoglutarate (AKG), which is an intermediate of the Krebs cycle, was shown to prevent lipid peroxidation in rats under chronic ethanol administration [6] and cyanide treatment [7], as well as to stabilize redox homeostasis in aged mice [8]. In addition, exogenous AKG displayed neuroprotective effects in the experimental ischemia of hippocampus [9], inhibited H_2O_2 -induced hemolysis of erythrocytes [10] and alleviated developmental toxicity of ethanol on *Drosophila melanogaster* [11]. Similar effects were demonstrated for other keto acids such as oxaloacetate (OA) and pyruvate which are intermediates in Krebs cycle and aerobic glycolysis, respectively. In part, exogenous OA reduced iron-induced lipid peroxidation in rat brain in vitro [12], and both OA and pyruvate could moderate the level of lipid peroxidation products in H_2O_2 -induced cataract [13]. Exogenous pyruvate was revealed to protect against oxidative damages to the eye lens in vitro [14], isolated and cellular DNA [15] and H_2O_2 -induced cytotoxicity [16–18]. Moreover, the ability of the alpha-keto acids (KAs) to scavenge H_2O_2 generated in cell culture medium upon the addition of autooxidizable compounds such as ascorbate, or polyphenols such as epigallocatechin gallate was proposed [19–21]. It was assumed that keto acids could directly react with H_2O_2 and in such a way neutralize this oxidant. Along with this, the authors did not exclude more complex reactions between KAs and hydrogen peroxide or effects of the presence of other redox-active compounds in cell culture medium due to difference in H_2O_2 -scavenging activity of different keto acids [21]. Additionally, the protective action of alpha-keto acids under various stressful challenges allows hypothesizing that the antioxidant effects of KAs can be explained not by their H_2O_2 -scavenging activity.

This work aimed to carry out comparative assessment of antioxidant activity of pyruvate, oxaloacetate and alpha-ketoglutarate using different methodologies in vitro and in vivo. In experiments in vivo, the effects of the alpha-keto acids on stress resistance to different oxidants in baker's yeast *Saccharomyces cerevisiae* and fruit fly *D. melanogaster* were analyzed.

Materials and Methods

Chemicals

Ethylenediamine-tetraacetic acid (EDTA), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt

(ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), xylenol orange, sodium pyruvate, oxaloacetic acid, sorbitol, thiobarbituric acid, ferrous sulfate and menadione were obtained from Sigma-Aldrich Corporation (USA). All other reagents were from local suppliers (Ukraine), and they were of analytical grade.

Assays of antioxidant activity of KA in vitro

Determination of total antioxidant activity by phosphomolybdate method

The total antioxidant activity (TAA) of keto acids was evaluated by the phosphomolybdenum method described by Prieto et al. [22]. Briefly, 0.2 ml of KA at different concentrations was mixed with 2 ml of reagent solution (0.6 M sulfuric acid, 28 mM potassium phosphate and 4 mM ammonium molybdate). The tubes containing reaction solutions were incubated at 95 °C for 90 min. The absorbance of the mixtures was then measured at 695 nm using spectrophotometer Spekol 211 (Carl Zeiss Jena, Germany) after cooling to room temperature. Ascorbic acid in a range from 5 to 1120 μ M was used for calibration curve building. The antioxidant activity of KA was expressed as μ M equivalents of ascorbic acid.

Assay of reducing power ability

The ability of KAs to reduce ferric ions was determined by the formation of Perl's Prussian blue at 700 nm accordingly to the method described in [23]. For this, 0.25 ml of KA at different concentrations was mixed with 0.5 ml of 200 mM potassium phosphate buffer (KPi, pH 6.6) and 0.5 ml of 1 % aqueous potassium hexacyanoferrate [$K_3Fe(CN)_6$] followed by incubation at 50 °C in water bath for 30 min. After incubation, 0.5 ml of 10 % TCA was added to terminate the reaction. The upper portion of the solution (0.5 ml) was mixed with 1 ml distilled water, and 0.1 ml of 0.01 % $FeCl_3$ solution was added. The reaction mixture was left for 10 min at room temperature, and the absorbance was measured at 700 nm. Ascorbic acid in a range from 5 to 1120 μ M was used as a standard, and the results were expressed as μ M equivalents of ascorbic acid.

ABTS⁺ scavenging activity

The ability of KAs to scavenge ABTS⁺ radical cation was compared to Trolox standard [24]. The ABTS⁺ radical cation was pre-generated by dissolving 11 mg ABTS in 2 ml of solution of 2 mM H_2O_2 in 30 mM acetate buffer (pH 3.6) to final ABTS concentration of 10 mM and incubating for 1 h in the dark at room temperature until characteristic color of ABTS⁺ appeared. The incubating samples were

prepared by mixing 150 μl of 0.4 M acetate buffer solution (pH 5.8), 15 μl of 10 mM ABTS⁺ solution and 10 μl of KA at different concentrations. The rate at which absorbance of ABTS⁺ cation radical at 414 nm dropped in the presence of KA was monitored using Labsystem Multiskan MCC/340. Solutions of Trolox within a concentration range from 9 to 90 μM were used as standards for calibration curve.

H₂O₂ scavenging

For determination of H₂O₂-scavenging activity, mixture containing 10 mM H₂O₂ and different concentrations of KA in 50 mM KPi (pH 7.0) was incubated for 30 min at room temperature. After incubation, the samples were diluted and 200 μl of the samples was mixed with 1.8 ml FOX reagent (250 μl FeSO₄, 25 mM H₂SO₄, 100 μl xylenol orange and 100 mM sorbitol) according to previously described FOX method [25]. The reaction mixture was then incubated at room temperature for 30 min. The absorbance of the ferric–xylenol orange complex was measured at 580 nm. The percentage of hydrogen peroxide scavenged by KA was calculated as follows: %Scavenged [H₂O₂] = [(A₀ – A_X)/A₀] × 100, where A₀ was the absorbance of the control (without KA) and A_X was the absorbance in the presence of respective KA.

Iron chelating

The 1,10-phenanthroline method [26] was used to examine the iron-chelating properties of KAs. The mixture containing freshly prepared FeSO₄ (150 μM) and different concentrations of KA was allowed to react for 5 min at room temperature. After that, 1,10-phenanthroline solution was added to determine free Fe²⁺ which did not react with KA. The absorbance was measured at 510 nm, and EDTA was used as a positive control. The values were expressed in percentage of control determined in the absence of KA.

Hydroxyl radical-scavenging activity

The hydroxyl radical-scavenging activity of KAs was measured by the deoxyribose assay according to Halliwell et al. [27]. The assay is based on quantification of the degradation product of 2-deoxyribose followed by condensation with thiobarbituric acid (TBA). Hydroxyl radicals were generated in the Fe³⁺–ascorbate–EDTA–H₂O₂ system (Fenton reaction). The reaction mixture contained 2.8 mM deoxyribose, 20 mM KPi buffer (pH 7.4), 100 μM freshly prepared FeCl₃, 100 μM EDTA, 1 mM H₂O₂, 100 μM ascorbic acid and varying concentrations of tested KA. The tubes containing reaction solutions were incubated at 37 °C for 60 min. After incubation, 1 ml 2.8 % TCA was added

to 0.5 ml of the reaction mixture to determine the reaction, followed by the addition of 1 ml 1 % aqueous TBA solution and the mixture was incubated at 90 °C for 15 min to develop the color. After cooling, the absorbance was measured at 532 nm against an appropriate blank mixture (without KA). Inhibition percentage was evaluated by comparing the test and blank solutions.

Assays of antioxidant activity of KA in vivo

Yeast oxidative stress assay

The *S. cerevisiae* strain YPH250 (wide type, *MATa trp1- Δ 1 his3- Δ 200 lys2-801 leu2- Δ 1 ade2-101 ura3-52*) used in this study was kindly provided by Dr. Yoshiharu Inoue (Kyoto University, Japan). Yeast cultures were grown in liquid medium YPD (1 % yeast extract, 2 % peptone and 2 % glucose) using an orbital shaker at 28 °C and 175 rpm, with 5:1 of flask volume/medium ratio. The cells from experimental cultures were collected after 18-h growth by centrifugation (5 min, 3000 g), washed with 50 mM KPi buffer (pH 7.0) and resuspended in equal volume of the same buffer. Then, the aliquots of experimental suspensions were exposed to 10 mM H₂O₂, 2 mM FeSO₄ or 100 mM menadione in the absence or the presence of 10 mM alpha-keto acids followed by their incubation at 28 °C for 30 min. Control samples were incubated under the same conditions but without stressors. Cell survival after stress exposure was evaluated by plating in duplicate on solid YPD medium after proper dilution. Plates were incubated at 28 °C for 72 h and the colony-forming unit (CFU) numbers counted. Tolerance was expressed as percentage of survival relative to control viability [28].

Testing of resistance of adult D. melanogaster flies to menadione and H₂O₂

Wild-type Canton S flies were used in the experiments. Stock flies were kindly provided by Bloomington Stock Center (Indiana University, USA). All fly cultures were kept at 25 °C, 55–60 % humidity in a 12-h dark/light cycle. Parental populations of flies were maintained on yeast–corn–molasses media containing 7.5 % molasses, 5 % yeast, 6 % cornmeal, 1 % agar and 0.18 % methylparaben to inhibit mold growth [29]. For experiments, flies were reared starting from egg stage on the medium containing 5 % sucrose, 5 % yeast, 1 % agar and 0.18 % methylparaben. Newly eclosed flies were transferred into the flasks with the respective experimental foods and held for 2 days. Two-day-old flies were mildly anesthetized with carbon dioxide gas and separated by sexes, and ten individuals were transferred into empty vials for 2 h of starvation. After starvation, flies were transferred into vials containing

folded and rammed strips (2.4×12 cm) of four-layer cellulose filter paper soaked with 0.8 ml 5 % sucrose solutions containing additionally (1) 20 mM menadione, (2) mixture of 20 mM menadione and 10 mM AKG, (3) 1 M H_2O_2 , and (4) mixture of 1 M H_2O_2 and 1 M AKG. Survivors were counted during 72 h of exposure at defined time intervals, and values were expressed in percentages of survived flies.

Statistical analysis

Experimental data are given as the mean value \pm the standard error of the mean (SEM) of 4–6 independent experiments. Statistical analysis was performed using one-way ANOVA followed a two-tailed Student's *t* test for comparison between two means or a Dunnett's test to compare multiple experimental treatments to the single control value with the use of the Mynova program (version 1.3). A difference was considered statistically significant when $P \leq 0.05$.

Results and discussion

Antioxidant properties of keto acids in vitro

The alpha-keto acids such as pyruvate, AKG and OA are important intermediates in cellular metabolism, particularly in energetic processes. During last decades, their protective role against various stressful factors accompanied by oxidative stress development was demonstrated in different models in vivo and in vitro [6, 7, 10–18]. The detailed mechanisms of such beneficial effects of KAs are not fully established, although their antioxidant role is offered. In this study, we compared the antioxidant activity of aqueous solutions of the above-mentioned alpha-keto acids, namely pyruvate, AKG and OA, using different methods. The results in vitro are represented in Figs. 1 and 2.

The tested KAs showed relatively high total antioxidant activity (TAA) measured by phosphomolybdate method with using ascorbic acid as a standard (Fig. 1a). The method is based on reduction of Mo(VI) to Mo(V) by the antioxidant compound and the formation of green phosphate/Mo(V) complex with a maximum absorption at 695 nm [22]. The TAA of KAs was significantly lower than in ascorbic acid, but showed a clear concentration dependency. The tested keto acids differed in their TAA (Fig. 1a). For instance, TAA of 10 mM solutions of pyruvate, OA and AKG was 79 ± 20 , 132 ± 9 and 229 ± 30 μ M ascorbic acid equivalents, respectively. In general, analyzing the regression equation and comparing coefficient of slope (Fig. 1a), we can conclude that TTA of AKG solutions was about 2.8 and 1.9 times higher than ones in pyruvate and OA, respectively. In turn, OA had 1.4-fold higher TAA than

pyruvate. Thus, efficiency of KA can be placed in the following order by TAA: AKG > OA > pyruvate.

The evaluation of antioxidant properties of KA solutions was also conducted by the method based on the reduction of colored ABTS^{•+} cation. The decolorization of ABTS^{•+} as the percentage inhibition of absorbance at 414 nm was determined as a function of concentration and time and calculated relative to the reactivity of Trolox as a standard under the same conditions [24]. The KAs showed ABTS^{•+} reducing activity in the manner dependent on type and dose of used acid (Fig. 1b). Unlike TAA measured by phosphomolybdate method, the highest ability to scavenge ABTS^{•+} radical was observed for OA and the lowest one was for AKG (with value of coefficient linear regression 15.5 vs. 3.85, respectively). The keto acids demonstrated very weak reducing ability measured by Fe^{3+}/Fe^{2+} conversion [23] comparing with ascorbic acid (Fig. 1c). In this case, clear dose dependency was not observed.

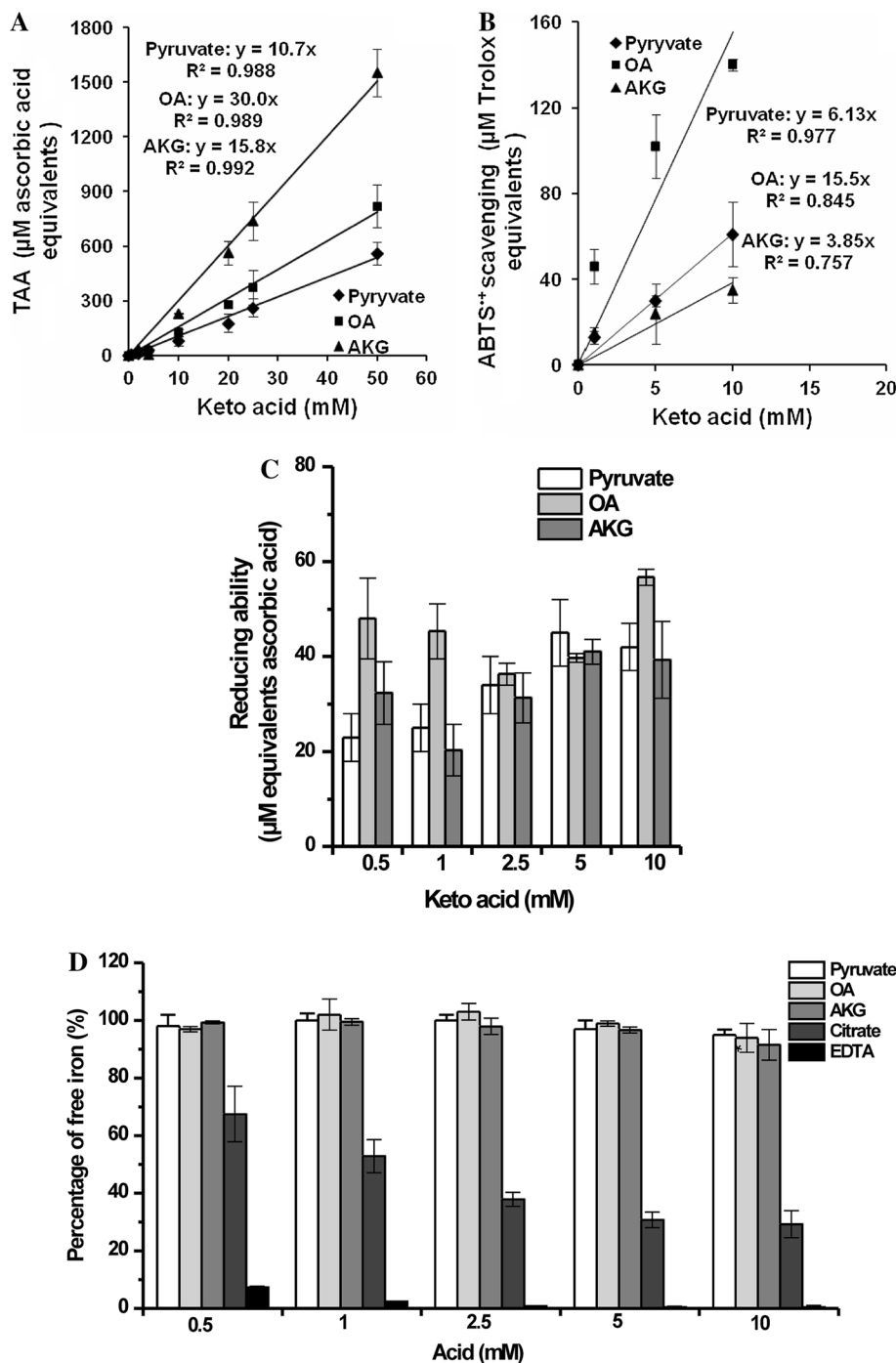
In total, results of three used methods of determination of TAA suggest that pyruvate, OA and AKG possess the antioxidant activity in vitro and are able to scavenge free radicals and to reduce oxidized molecules demonstrating specificity for the oxidized compounds.

We did not find the chelating ability of tested KAs toward free iron (II) by using the broad range of KA concentrations in contrast to EDTA, a well-known iron-chelating compound, and citrate with moderate chelating activity (Fig. 1c). These results suggest that pyruvate, OA and AKG are unable to bind free iron efficiently. It should be noted that this is not consistent with other data reported the chelating ability of OA and AKG [12]. We suggest that the structure of KAs does not allow them to form inactive complexes with iron (II).

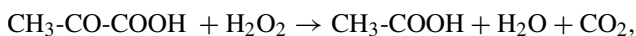
Figure 2a demonstrates hydroxyl radical-scavenging ability of KAs measured as an ability of the compounds to inhibit $\cdot OH$ -mediated deoxyribose degradation in the Fe^{3+} -ascorbate-EDTA- H_2O_2 system [27]. As in the case of total antioxidant activity, KAs demonstrated a concentration-dependent manner in the inhibition of deoxyribose degradation. Herewith, all three KAs did not show a powerful $\cdot OH$ -neutralizing activity. The regression equation suggests that AKG can more effectively scavenge $\cdot OH$ or prevent its formation than other KAs, and all KAs may be placed in the order of decreasing their $\cdot OH$ -neutralizing ability as follows: AKG > pyruvate > OA.

Previously it was proposed that some KAs can non-enzymatically react with H_2O_2 , thereby neutralizing the latter [19–21]. To check it and to compare the H_2O_2 -scavenging activity of pyruvate, OA and AKG, their solutions at different concentrations in 50 mM KPi buffer (pH 7.0) were incubated with 10 mM H_2O_2 for 30 min at room temperature. The concentration of H_2O_2 decreased in reaction medium during incubation with alpha-keto acids in dose-dependent

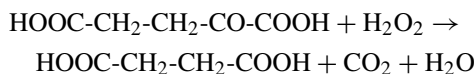
Fig. 1 Antioxidant activity and iron-chelating ability of aqueous solutions of alpha-keto acids measured by different methods. **a** TAA by phosphomolybdate method, **b** ABTS⁺ scavenging activity, **c** reducing activity measured as the ability to reduce ferric ions with formation of Perl’s Prussian blue, **d** iron-chelating activity by 1,10-phenanthroline method. Absorbance of complex between free Fe²⁺ and 1,10-phenanthroline in the absence of KA was set as 100 %. EDTA and citrate were used as positive controls. Explanations are in the text. Data are mean ± SEM, n = 4–5



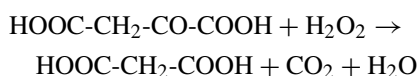
manner (Fig. 2b). While H₂O₂ level at initial 10 mM decreased by 9.8–9.5 mM at the presence of 0.5 mM KA, it almost disappeared at incubation with 10 mM KA at percentage of scavenging of 96–100 %. Thus, all tested alpha-keto acids displayed a good H₂O₂-scavenging ability that is consistent with previous reports [15, 16, 19–21]. The reaction of H₂O₂ with pyruvate is often presented as [30]



while the reaction of AKG with H₂O₂ was proposed to generate succinate, carbon dioxide and water [10, 21]:



OA seems to react with H₂O₂ as follows:



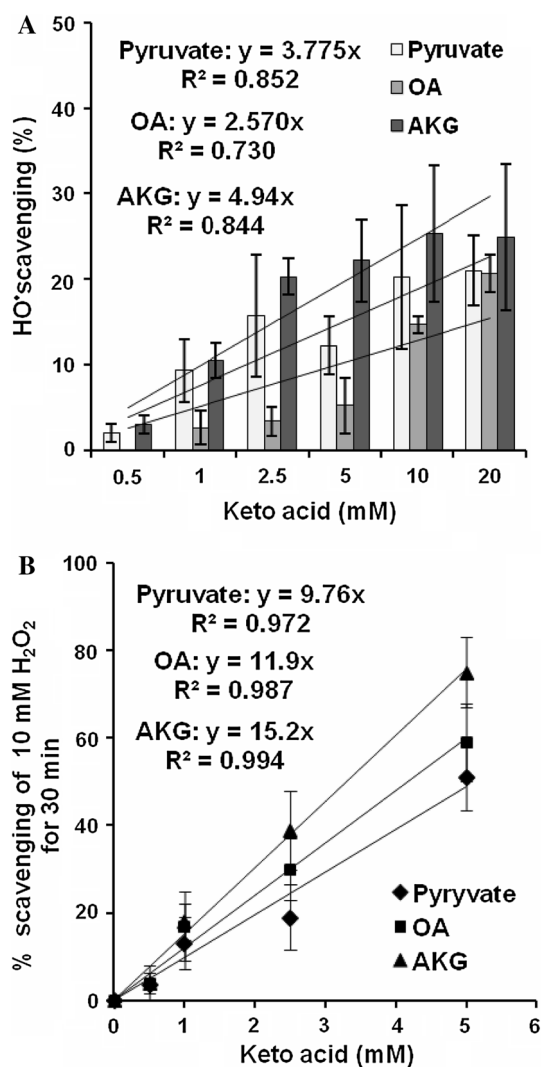


Fig. 2 Hydroxyl radical-scavenging (a) and H_2O_2 -scavenging (b) activities of alpha-keto acids: **a** hydroxyl radicals generated by the Fe^{3+} -ascorbate-EDTA- H_2O_2 system and reacted with 2-deoxy-2-ribose to produce malondialdehyde (MDA)-like product in the absence or presence of KAs. This compound forms a pink chromogen upon heating with TBA at low pH; **b** pyruvate, AKG and OA at different concentrations were incubated with 10 mM H_2O_2 for 30 min at room temperature, and then H_2O_2 level in the samples was measured by FOX method. Absorbance of ferric-xylene orange complex formed in the absence of AKG was set to 100 %. Data are mean \pm SEM, $n = 5$

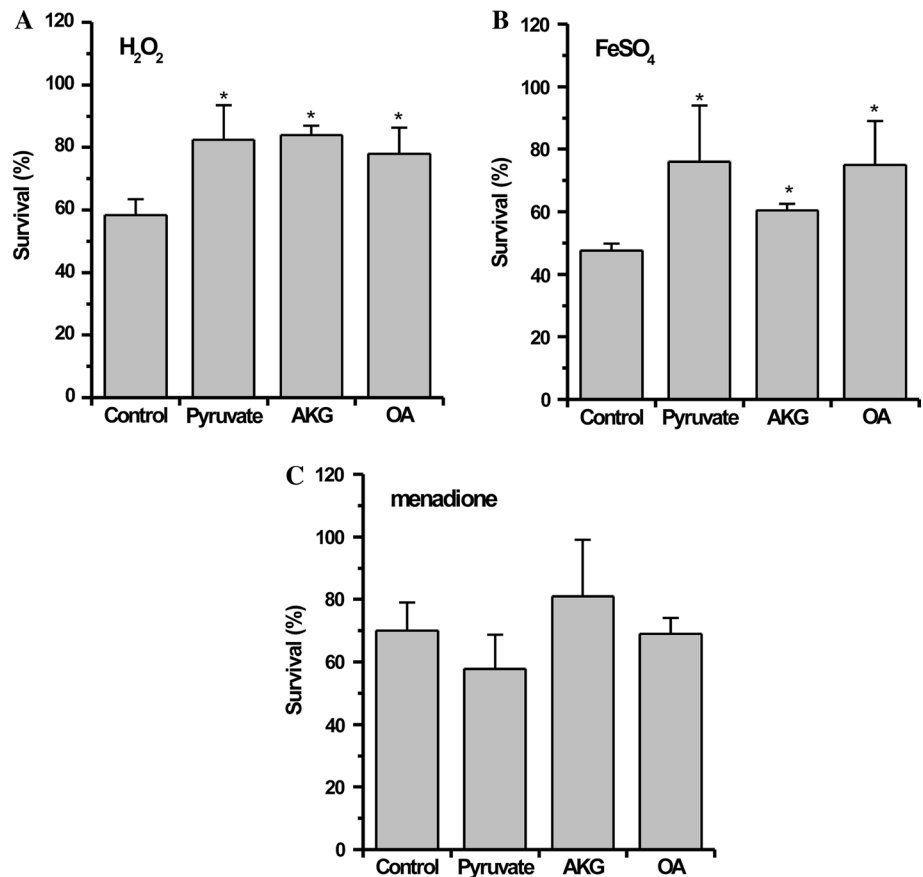
At the same time, analysis of regression equation allows to conclude that the studied KAs differ in H_2O_2 -scavenging activity. The highest H_2O_2 -scavenging activity was detected for AKG, while pyruvate possessed the lowest activity. It seems that interaction of keto acids with H_2O_2 can be far complex processes than it was considered before. Similar results were obtained in the studies reported the ability of alpha-keto acids to neutralize H_2O_2 generated in cell culture medium upon the addition of autooxidizable

compounds such as ascorbate [19–21]. However, in contrast to those results showed the most effective H_2O_2 -scavenging ability for pyruvate, in our experiments, pyruvate was less effective than H_2O_2 scavenger comparing with AKG and OA. Obviously, this might result from difference in the composition of the reaction medium which could affect the H_2O_2 -scavenging activity of keto acids. Comparing our data on reducing ability (Fig. 1c) and H_2O_2 scavenging (Fig. 2b) along with $HO\cdot$ scavenging (Fig. 2a), we may assume that $HO\cdot$ scavenging activity of KAs might be mostly a result of neutralization of H_2O_2 and less an effect of recycling of oxidized iron. AKG with the highest H_2O_2 -scavenging activity demonstrated also the most pronounced ability to decrease $HO\cdot$ level in the Fenton system, whereas OA with moderate H_2O_2 -scavenging activity and higher ability to reduce to oxidized iron needed for Fenton reaction possessed the lower ability to prevent effects of hydroxyl radical.

Antioxidant properties of keto acids in vivo

The antioxidant properties of pyruvate, OA and AKG observed above in vitro allow assuming that these alpha-keto acids can be effective protectants against oxidants in vivo. To compare protective role of the KAs in vivo, we evaluated survival of yeast *S. cerevisiae* cells treated with lethal concentrations of different redox-active compounds at the presence or the absence of KA. Hydrogen peroxide, Fe^{2+} (in the form of $FeSO_4$) and menadione were used as oxidant agents [28, 31, 32]. Survival of yeast cells in control cultures (without KAs) was substantially reduced upon treatment with these compounds and amounted 58, 48 and 70 % in the case of 10 mM H_2O_2 , 2 mM Fe^{2+} and 100 mM menadione exposures, respectively (Fig. 3a–c). The combined treatment with stressors and 10 mM KAs alleviated yeast sensitivity to hydrogen peroxide and Fe^{2+} , but did not affect yeast resistance to menadione. All studied keto acids showed similar effects. Toxicity of Fe^{2+} is connected, at least partly, by its interaction with H_2O_2 in Fenton reaction with generation of highly reactive hydroxyl radical [27]. Menadione is a well-known superoxide anion-generating compound [31, 32]. The obtained results suggest that protective action of AKG is to some extent specific, and it is manifested against the factors involving H_2O_2 -mediated effects. This conclusion is confirmed by observed above high H_2O_2 -scavenging ability of KAs in vitro. In the case of H_2O_2 exposure, alpha-keto acids seem directly to neutralize this oxidant alleviating its toxic action on yeast cells. At the same time, under treatment with $FeSO_4$, the protective effect of KAs may be realized due to their reaction with intracellular H_2O_2 preventing its interaction with Fe^{2+} . The iron-chelating ability of KA should be excluded because we did not detect this ability in vitro.

Fig. 3 Survival of *S. cerevisiae* YPH250 cells treated with 10 mM H₂O₂ (a), 2 mM FeSO₄ (b) or 100 mM menadione (c) in the presence of alpha-keto acids. Cells growing exponentially in YPD medium were collected and resuspended in 50 mM potassium phosphate buffer (pH 7.0). Cell suspensions were then exposed to different stressors for 1 h at 28 °C with adding or without respective KA (pyruvate, OA, AKG). Cell survival without treatment with stressors was accepted as 100 %. Data are mean ± SEM, *n* = 5–6. *Significantly different from respective control values with *P* < 0.05 using Student's *t* test



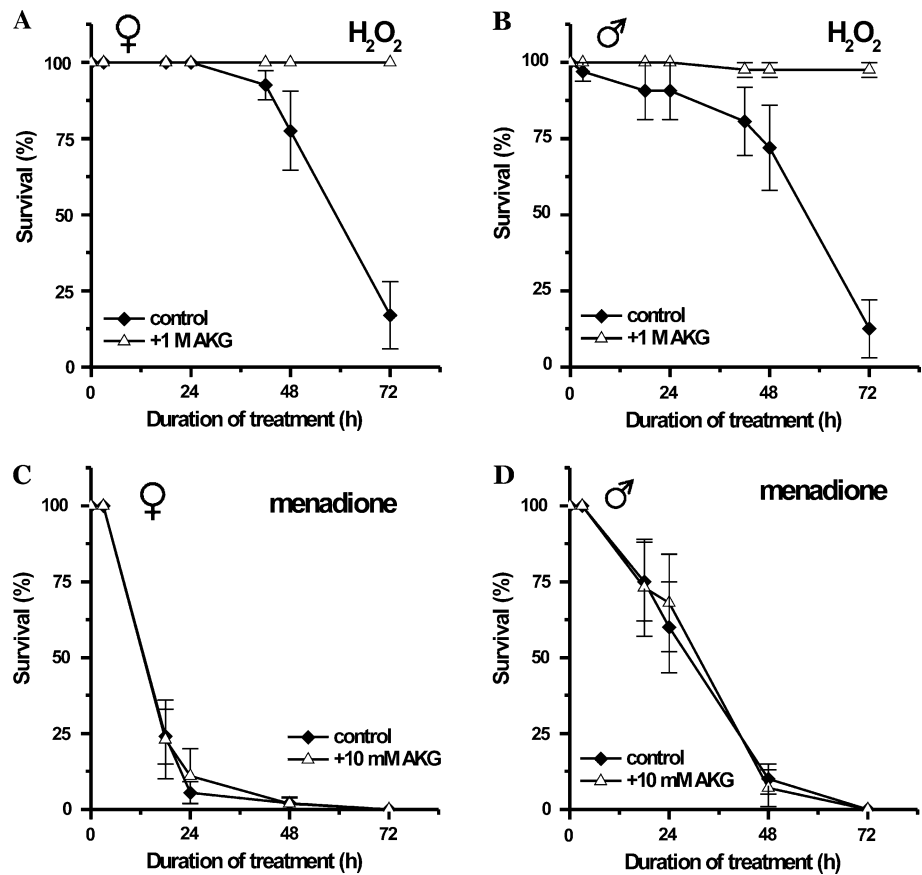
The beneficial effects of pyruvate, OA and AKG attributed to its antioxidant properties have been previously demonstrated with other model systems *in vivo* and *in vitro* [6, 10, 12–18]. The protective effects of alpha-keto acids against menadione-induced DNA injury and cytotoxicity in the human breast cancer cell line were reported among these studies [15]. We did not observe protective effects of KAs against menadione on yeast cells.

To exclude the peculiarities of yeasts as a model regarding the received results, we checked the protective ability of KA against menadione toxicity on another model, a fruit fly *D. melanogaster*. For testing, we used only AKG. In parallel, we conducted the test on the sensitivity of fruit flies to H₂O₂ with and without addition of AKG. The maintenance of 2-day-old *D. melanogaster* females and males on 5 % sucrose solution supplemented with 1 M H₂O₂ decreased fly viability over time, and females demonstrated slightly higher survival for first 42 h of treatment comparing with males (Fig. 4a, b). The percentage of alive females maintained on H₂O₂ for 42 h was 92.5 and 72.5 % in males (*P* < 0.05). The supplementation with 1 M AKG almost completely neutralized the negative effects of 1 M H₂O₂ on fly viability. Upon combined treatment with 1 M H₂O₂ and 1 M AKG, 100 % of females and 97.5 % of males remained alive at 72 h of incubation. In the case of menadione

treatment, the flies showed much higher sensitivity to this oxidant than to hydrogen peroxide, and all flies died over incubation for 72 h (Fig. 4c, d). The combined administration of 20 mM menadione and 10 mM AKG did not alleviate the toxic effects of menadione on flies. Thus, the results of these tests were generally similar to the ones observed on yeasts, namely KAs showed the protective effects against H₂O₂, but not menadione. It confirmed our previous assumption toward specificity of antioxidant action and, as a result, protective effects of KA against different oxidants. As effective H₂O₂ scavengers, KA can prevent, first of all, H₂O₂-mediated toxic effects (Fig. 4).

In summary, the obtained results demonstrate that natural alpha-keto acids, such as pyruvate, OA and AKG, possess the antioxidant activity *in vitro* and *in vivo*. *In vitro*, the KA showed a good H₂O₂-scavenging activity, but less effectively reduced other free radicals or oxidized compounds. Alpha-ketoglutarate was more effective scavenger of H₂O₂ and HO[•] *in vitro* comparing with other keto acids. In line with experiments *in vitro*, the tested KAs effectively prevented the death of yeast *S. cerevisiae* cells and adult *D. melanogaster* flies under exposure to H₂O₂. At the same time, the protective effects were absent in the case co-treatment with menadione, which is superoxide anion-generating compound. It seems that antioxidant action of KA is more manifested to

Fig. 4 Time survival of 2-day-old *D. melanogaster* Canton S flies under treatment with 1 M H_2O_2 or mixture of 1 M H_2O_2 and 1 M AKG (a, b), 20 mM menadione or mixture of 20 mM menadione and 10 mM AKG (c, d). Data are presented as the mean \pm SEM of four independent experiments with 20 flies per sex



H_2O_2 -mediated effects. The results suggest that the tested alpha-keto acids can be considered as natural antioxidants for preventing of ROS-mediated toxicity in living organisms under different stress conditions accompanying by oxidative stress development. The keto acids possess a significantly weaker antioxidant capacity (~100-fold lower) than well-known antioxidants like vitamins C and E. That capability of keto acids may be very useful because unlike vitamins, the keto acids can be added to a diet in large amounts without a risk of side effects. For instance, AKG is usually supplemented in average dose of ~1–2 g per kilogram of body mass daily to study its benefit effects in mice or pigs [8, 9, 33]. In experiments studying effects of keto acids on training efficiency and athletic performance, the volunteers consumed on average 5–25 g of AKG or pyruvate per day [34, 35]. Such diet enriched with keto acids makes possible exhibiting of their antioxidant properties. It seems that the keto acids as antioxidant supplements can be useful upon poisoning with different drugs, heavy metals, oxidants and other xenobiotics in which metabolism is connected with ROS formation. In support of this, the ability of AKG to prevent oxidative modification of biomolecules was observed earlier under treatment of model animals with ethanol [6], sodium valproate [36], sodium nitroprusside and cyanide [7]. The antioxidant defense of living organisms declines with age,

and it is supposed to be a cause of chronic oxidative stress, which leads as a result to development of many pathological states and diseases. Consumption of supplements with keto acids can diminish manifestations of age-associated oxidative stress, thereby preventing numerous health disturbances. In line, several studies reported benefit effects of alpha-keto acids in anti-aging therapy: Alpha-ketoglutarate stabilized redox homeostasis and improved arterial elasticity in aged mice [8], and pyruvate peels were shown as a safe and efficient treatment for moderate facial skin aging [37]. In addition, it was shown recently that AKG and oxaloacetate supplementation prolonged lifespan of nematode *Caenorab-dilis elegans* in dose-dependent manner, but the mechanisms responsible for this effect were proposed to be rather complicated and most likely do not involve direct antioxidant mode of action of keto acids [38, 39]. In general, the natural alpha-keto acids not only play metabolic roles, but possess many other useful properties, including antioxidant and detoxification ones, making them perspective tools for many therapeutic interventions.

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Compliance with ethical standards

Conflict of interest None.

Compliance with Ethics Requirements This article contains studies with invertebrates and does not contain any studies with human or other animal subjects.

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